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# The dorsoventral polarity of the presumptive limb is determined by signals produced by the somites and by the lateral somatopleure

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## SUMMARY

**When it first appears at stage HH16, the wing bud is already polarized along the dorsoventral axis. To study the mechanisms leading to the establishment of its dorsoventral polarity, we decided to focus our attention on an earlier stage (HH13). Using the quail-chick chimera system, we first show that the presumptive wing mesoderm occupies the medial half of the somatopleure at the level of somites 15-20. The corresponding ectodermal area, however, will only give rise to the apical ectodermal ridge. The rest of the limb bud ectoderm originates from the ectoderm overlying the paraxial and the intermediate mesoderms for its dorsal aspect and the lateral somatopleural mesoderm for its ventral aspect. We next used five experimental paradigms to show that the dorsoventral polarity of the presumptive limb is determined by its environment. Thus, presumptive limb regions flanked on two sides by rows of somites give rise to bidorsal limb buds, indicating that the somites produce a dorsalizing factor. In addition, insertion of filters laterally to the presumptive limb region also results in bidorsal limb buds, suggesting that the lateral somatopleure produces a ventralizing factor. We propose a model in which the polarizing activity of these two signals is mediated by the morphogenetic movements of the presumptive dorsal and ventral ectoderms, which carry the dorsoventral information over the limb bud mesenchyme.**

Key words: limb bud, somites, dorsoventral axis, chick, quail, induction

## INTRODUCTION

The vertebrate limb develops along three axes: anteroposterior (AP), dorsoventral (DV) and proximodistal. Most of the availaible information concerning the patterning of limb structures along these axes has come from studies carried out in chick embryos which can be easily manipulated in ovo (for a review, see Tickle and Eichele, 1994). The first indication of the development of the chick wing bud is a condensation of somatopleural mesoderm opposite somites 15-20 at stage HH16 (Hamburger and Hamilton, 1951). A few hours later, at the end of stage HH18, a thickening of the distal ectoderm, called the apical ectodermal ridge (AER), is induced by the mesoderm at the interface between the dorsal and the ventral sides of the wing bud (Kieny, 1960; Saunders and Reuss, 1974; Carrington and Fallon, 1984; Todt and Fallon, 1984). The AER is an important signaling center, which keeps the underlying mesenchyme undifferentiated and in a proliferative state (Summerbell et al., 1973). If the AER is surgically removed, growth stops and the limb is truncated along the proximodistal axis (Saunders, 1948). Before the appearance of the AER, the DV interface is not morphologically recognizable but can be defined by the expression of *Fgf-8* detectable from stage HH16 in a strip of ectodermal cells which have been suggested to give rise to the AER (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996).

As early as stage HH15/16, dorsal and ventral aspects of the limb bud can be distinguished by different molecular markers. For instance, *Wnt-7a* (Dealy et al., 1993; Riddle et al., 1995) and *En-1* (Davis et al., 1991; Gardner and Barald, 1992) are expressed in the dorsal and in the ventral sides of the limb bud ectoderm, respectively. The *Wnt-7a* expression domain is excluded from the AER but the *En-1* expression domain reaches the apex of this structure up to its midline. The factors patterning the limb DV polarity have long been the subject of investigation. It was established in the 1970s that this process involves tissue interactions between the two initial limb bud ectodermal and mesodermal components. Experiments carried out in the chick embryo showed that from stage HH15/16 on, the ectoderm is able to impose its polarity on the limb mesodermal rudiment. Thus, when the limb bud mesenchyme is jacketed in limb bud ectoderm whose dorsoventral axis has been reversed, epidermal, muscular and skeletal components of the distal limb develop with the dorsoventral polarity of the ectoderm (MacCabe et al., 1974; Pautou, 1977; Geduspan and MacCabe, 1987). Moreover, *Wnt-7a* and *En-1* appear to play a key role in mediating the DV polarizing activity of the limb bud ectoderm, since in mice homozygous for a mutation in *Wnt-7a*, dorsal structures of the limb bud adopt a ventral identity (Parr and McMahon, 1995), whereas loss of *En-1* function results in dorsal transformations of ventral paw structures (Loomis et al., 1996). *Wnt-7a* produces at least some of its effect by inducing *Lmx-1* in the underlying dorsal mesenchyme (Riddle et al., 1995; Vogel et al., 1995).

In contrast to what has been observed after the appearance of the limb bud at stage HH15/16, reversal of the dorsoventral orientation of the somatopleural ectoderm before that stage does not affect the dorsoventral polarity of the chick limb bud (Geduspan and MacCabe, 1989). This led to the contention that initially the dorsoventral information might reside in the mesoderm before being transferred to the ectoderm, which then gains the ability to impose its polarity on that of the mesoderm. However, the conclusion that the somatopleural mesoderm contains the DV information remains controversial. Saunders and Reuss (1974) have observed that, as early as stage HH12, presumptive wing bud mesoderm transplanted to the flank (i.e. the region between the two pairs of limb buds) gives rise to limbs having the dorsoventral polarity of the grafts. This indicates that the presumptive limb already contains the DV information at that stage. In contrast, using a similar approach, Kieny (1971) observed that when the dorsoventral axis of the graft is reversed, the resulting supernumerary limbs have the same DV polarity as ipsilateral, non-manipulated ones. This suggests that the environment polarizes the presumptive limb field. In view of these conflicting results, the mechanisms leading to the establishment of the DV polarity of the presumptive limb and of the limb bud ectoderm remain undefined. For this reason we decided to further investigate when and how patterning of the dorsoventral axis of the presumptive wing bud takes place. Our purpose was to examine the relationship between the presumptive limb and its environment with respect to the establishment of the dorsoventral polarity. We first constructed a fate map of the different ectodermal and mesodermal territories which will compose the limb bud, at a stage preceding the limb bud's appearance (stage HH13). An unexpected finding was that, at this stage, the ectoderm cells covering the entire presumptive limb region only give rise to the AER. Using the information provided by this fate map, we then designed a series of experiments with the goal of changing the environment of the presumptive wing region. We show that, at stage HH13, the dorsoventral polarity of the wing somatopleure is not yet determined. We provide evidence that the polarization of the wing somatopleure results from the production of a dorsalizing factor by the somites and of a ventralizing factor by the lateral somatopleure which operate between stage HH13 and HH15.

## MATERIALS AND METHODS

### Eggs and microsurgery

Quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus*) eggs from commercial sources were incubated at 38°C until embryos reached late stage HH13 (20 somites). All surgical experiments involved the somatopleure (lateral plate) of the wing region, which is located between somites 15 and 20 (Wolff, 1936). In most experiments, strips of somatopleure were dissected. A micrometer was used to determine their mediolateral limits with respect to the lateral border of the Wolffian duct. Their anteroposterior limits corresponded to the limits of the wing region. In the experiments in which these strips were grafted in place of the neural tube, the notochord was left intact. In some cases, the ventral portion of the neural tube was also left in place. Whether the neural tube ablation was complete or not did not affect the results. Grafts of wing somatopleure, with or without the paraxial structures, to the flank of stage-matched embryos were performed as described by Saunders and Reuss (1974). Briefly, a lesion was made in the flank ectoderm on the right side and a long pocket was tunneled between the ectoderm and the mesoderm of the flank somatopleure. The donor somatopleure, also taken from the right side, was then inserted in this pocket with its anteroposterior and dorsoventral axes inverted. The ectodermal layer covering the paraxial, the intermediate or somatopleural mesoderm was isolated either in $Ca^{2+}$- and $Mg^{2+}$-free PBS or after a 5 minutes incubation in 0.25% pancreatin (Gibco-BRL) in Tyrode. Embryos were collected either 14-24 hours (stage HH17-18) or 40-48 hours (stage HH20-23) after the surgery and processed for immunohistochemistry or in situ hybridization.

### Histology, immunohistochemistry and in situ hybridization

For immunohistochemistry and radioactive in situ hybridization, embryos were fixed in Carnoy's fluid, embedded in paraffin and cut in 5 μm transverse sections. In order to distinguish quail cells from chick cells, we used the monoclonal antibody QCPN (Developmental Studies Hybridoma Bank), which specifically recognizes a quail antigen present on all cell types, as described by Catala et al. (1996). Radioactive in situ hybridization was performed as previously described by Eichmann et al. (1993). Whole-mount in situ hybridization was performed according to Henrique et al. (1995). Stained embryos were processed for sectioning (50 μm) using a vibratome, after embedding in albumin-gelatin. The probes used in this study were *En-1* (Logan et al., 1992), *Fgf-8* (Crossley et al., 1996), *Lmx-1* (Riddle et al., 1995) and *Shh* (Riddle et al., 1993).

## RESULTS

### The respective origin of the AER and of the dorsal and ventral wing ectoderm

We used the quail-chick chimera system (Le Douarin, 1969) to map the mediolateral limits of the presumptive wing region at late stage HH13 (20 somites). This stage was chosen because it is the earliest at which the paraxial mesoderm of the wing region is segmented. The somites, the Wolffian duct and the somatopleure can therefore be clearly distinguished from each other. Strips of somatopleure, which had a width of 150 μm (approximately half of the somatopleure) and for which the medial limit was the lateral border of the Wolffian duct, were taken from the wing region of the chick and substituted by their quail counterparts ($n$=4) (Fig. 1A/experiment 1). The resulting limb buds were analyzed 40-44 hours later (stage HH21-22) for chimerism. The mesenchyme was almost completely composed of quail cells (Fig. 1B), except for the muscle precursor cells which arose from the chick lateral somites (Christ et al., 1974; Ordhal and Le Douarin, 1992). Unexpectedly, in the ectoderm, the quail cells were strictly restricted to the AER (Fig. 1C). They occupied its dorsoventral midline, whereas its periphery was composed of chick cells. In order to verify whether this restriction of quail cells to the AER is also the case earlier in development, we collected the chimeras produced by experiment 1, 24 hours after the procedure (stage HH18). At that stage the AER begins to appear (Todt and Fallon, 1984). The mesenchyme of these early chimeric limb buds is mainly composed of quail cells ($n$=4; Fig. 1D). In the ectoderm, however, these cells occupied only the whole thickened region which gives rise to the AER.

We conclude from these results that the mesodermal somatopleural component of the limb bud is entirely contained in the area transplanted in this experiment. We will hereafter designate this as zone W (for wing). It thus appears that,

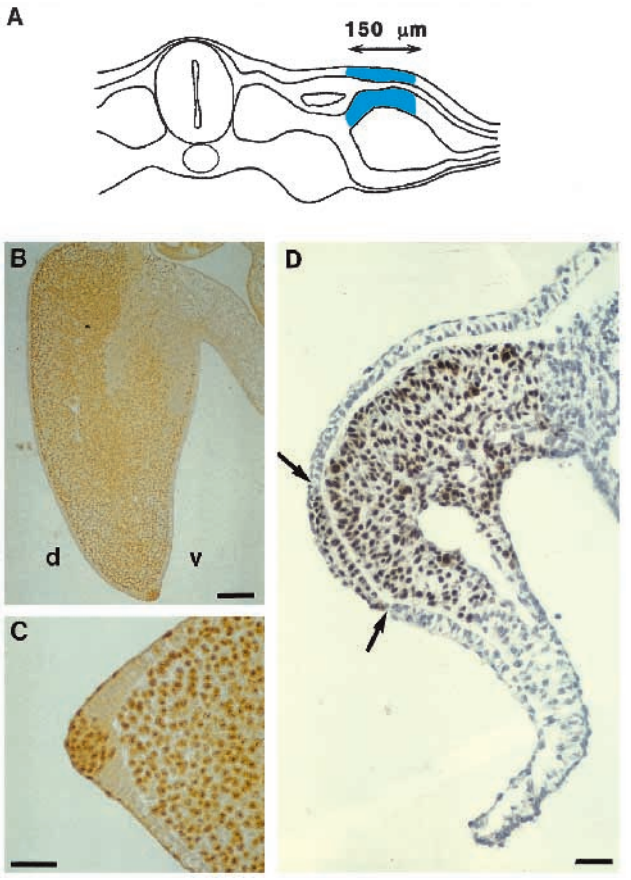


**Fig. 1.** Experiment 1. Orthotopic quail to chick graft of the medial somatopleure in stage-matched embryos. (A) Schematic transverse section in the wing region of a late stage HH13 embryo indicating the mediolateral limits of the graft. Its medial limit is the lateral border of the Wolffian duct and its width is 150 μm. (B) Transverse section of a stage HH22 limb bud resulting from the graft described in A. The quail cells are revealed by the anti-QCPN antibody (in brown). The mesenchyme is mainly composed of quail cells except for the muscle precursor cells derived from the chick host somites. (C) Higher magnification of the section shown in B. In the ectoderm, the quail cells are found around the dorsoventral midline of the AER. (D) Transverse section of a stage HH18 limb bud resulting from the graft shown in A. In the ectoderm, the quail cells occupy the thickened region which will give rise to the AER. Bars, 100 μm (B) and 30 μm (C,D).

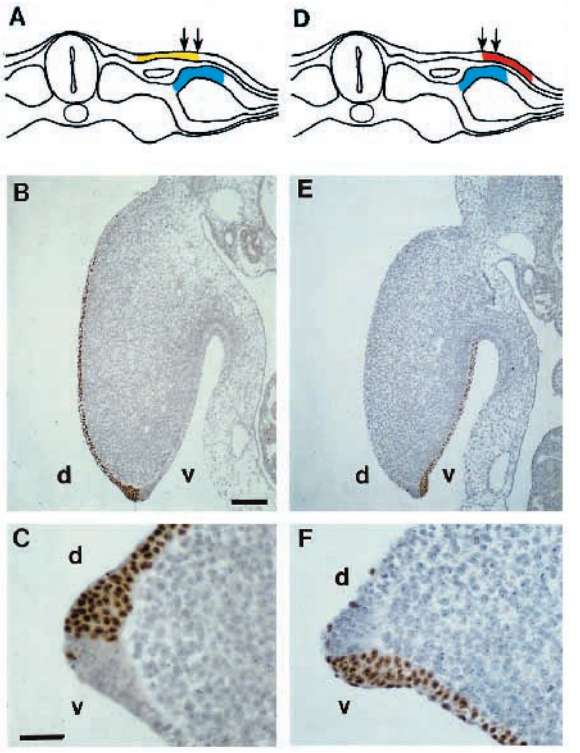


**Fig. 2.** Orthotopic quail to chick grafts of ectodermal strips overlying the intermediate and somatopleural mesoderms. (A,D) Schematic transverse sections in the wing region of a late stage HH13 embryo showing the mediolateral limits of the grafts (in yellow and in red) and the presumptive limb mesoderm (zone W, in blue). (A) Experiment 2. The medial limit of the graft is the lateral border of the somites. Its lateral limit is located near the middle of zone W (in yellow) within a region of 50-75 μm indicated by arrows. (B) Transverse section of a stage HH22 limb bud resulting from experiment 2. Almost all the dorsal ectoderm is composed of quail cells which are revealed by the anti-QCPN antibody (in brown). (C) Higher magnification of the section shown in B. The quail cells occupy the dorsal half of the AER. (D) Experiment 3. The medial limit of the graft is located near the middle of zone W, within a region of 50-75 μm indicated by arrows. The width of the graft is approximatively 200 μm. (E) Transverse section of a stage HH22 limb bud resulting from experiment 3. The ventral ectoderm is composed of quail cells. (F) Higher magnification of the section shown in E. The quail cells occupy the ventral half of the AER. Bars 100 μm (B,E) and 30 μm (C,F).

initially, at stage HH18, the DV interface is completely derived from the ectoderm overlying the presumptive wing region. With development of the AER, some cells, which originate either from the dorsal or the ventral ectoderm of the limb bud, are incorporated into the periphery of the AER.

It remained, therefore, to determine the origin of both the dorsal and the ventral ectodermal components of the wing. For this purpose, defined strips of ectoderm were isolated from the medial and the lateral sides of the zone W. In experiment 2, a strip of quail ectoderm covering the intermediate mesoderm and approximately the medial half of zone W was grafted isotopically in stage-matched chick embryos (Fig. 2A). In experiment 3, a strip of ectoderm which was 200 μm wide and for which the medial limit was approximately the middle of zone W was exchanged between quail and chick under the same conditions (Fig. 2D). The chimeras were analyzed at stage HH21-23. In experiment 2 ($n$=8), the quail cells were found on the dorsal side of the limb bud and occupied precisely the dorsal half of the ridge (Fig. 2B,C). The proximal third of the limb bud dorsal ectoderm was composed of chick cells derived from the ectoderm overlying the somites. In experiment 3 ($n$=5), the quail cells were found in the ventral ectoderm of the limb bud and reached the dorsoventral midline of the AER (Fig. 2E,F). We conclude that the dorsal ectoderm is derived from the ectoderm overlying the paraxial and the intermediate mesoderms and that the ventral ectoderm is derived from the lateral somatopleural ectoderm. In view of the fact that there is a margin of error when grafting into a region without morphological boundaries, it is remarkable that in both experiments 2 and 3 the boundary between the chick and quail cells

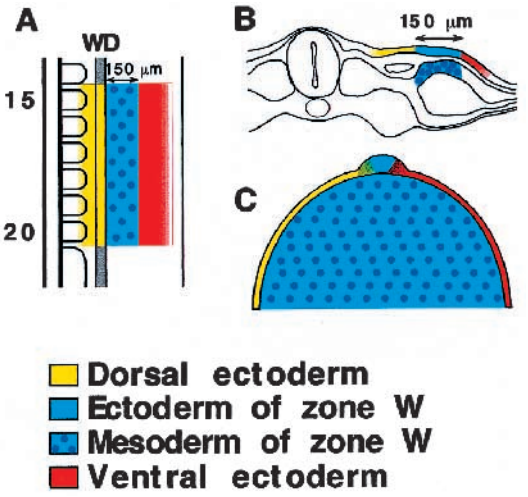


**Fig. 3.** Presumptive territories of the limb bud in late stage HH13 embryos (20 somites) as deduced from experiments 1, 2 and 3. (A) Scheme representing a dorsal view of the wing region which is located between somites 15 and 20. (B) Schematic transverse section in the wing region. (C) Schematic transverse section of a stage HH20 limb bud. (A-C). The medial somatopleure (zone W) gives rise to the limb bud mesenchyme and to the dorsoventral midline of the AER. The dorsal ectoderm (yellow) is derived from the ectoderm overlying the paraxial and intermediate mesoderms. The ventral ectoderm (red) is derived from the lateral somatopleural ectoderm. The dorsoventral interface is intially entirely composed of ectodermal cells originating form zone W. With further development, cells from the dorsal and ventral ectoderms are incorporated in the AER as shown by the areas of blended colors.

was always precisely the dorsoventral midline of the AER. This observation is consistent with our finding that, at late stage HH13, the cells which will give rise to the AER occupy a wide territory. Results of experiments 1, 2 and 3 are summarized in Fig. 3.

### The polarity of the dorsoventral axis is not determined at stage HH13

To determine whether the DV axis of the wing territory is established at HH13, we performed a 180° rotation of the ectoderm and mesoderm of zone W, which resulted in inversion of both the dorsoventral and anteroposterior axes (Fig. 4A). Manipulated embryos were collected 2 days later (stage HH21-22) and analyzed for the expression of *Lmx-1*, a marker of the limb bud dorsal mesenchyme. In all cases ($n$=5), *Lmx1* was found to be expressed dorsally, in the same domain as in control limbs (Fig. 4B). The growth of the manipulated limbs was oriented anteriorly and *shh*, a marker of the posterior mesenchyme, was found to be expressed anteriorly ($n$=5), showing that the anteroposterior polarity was reversed (Fig. 4C). We conclude that, at this stage, the dorsoventral polarity is not yet determined.

### A presumptive limb region flanked by two rows of somites gives rise to a bidorsal limb bud

We hypothesized that the paraxial structures could play a role in the induction of the dorsoventral polarity of the presumptive limb region. To test this possibility, we flanked the presumptive limb region with two rows of somites. Strips of somatopleure were taken from late stage HH13 quail embryos between somites 15 and 20. Some of these strips entirely contained the W area (Fig. 5, type A; $n$=7); some others were taken more laterally, from a region overlapping the W area and the lateral somatopleure (Fig. 5, type B; $n$=11); and in a last series, they came from the region lateral to the W area (Fig. 5, type C; $n$=9). The strips were grafted into stage HH13 chick

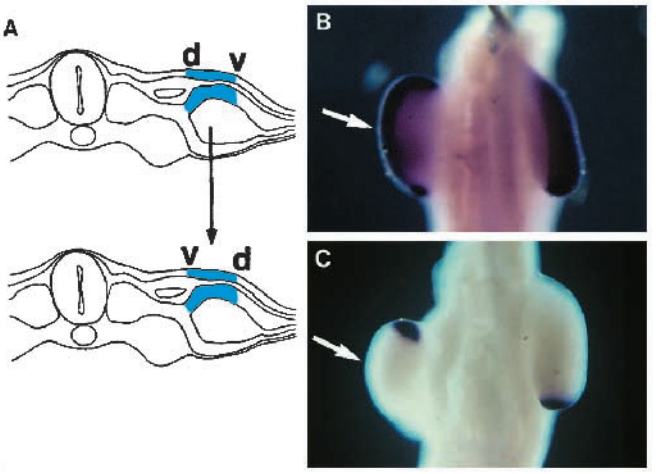


**Fig. 4.** Rotation of the presumptive wing mesoderm and overlying ectoderm at late stage HH13. (A) Schematic transverse section showing that a 180° rotation of zone W results in an inversion of the prospective dorsoventral polarity. (B,C) An operation was performed on the right side, as indicated by the arrow. Expression of *Lmx-1* (B) and *Shh* (C) shows that the dorsoventral polarity is maintained, whereas the anteroposterior axis is inverted. Note that, accordingly, the manipulated limb buds grow in an anterior direction.

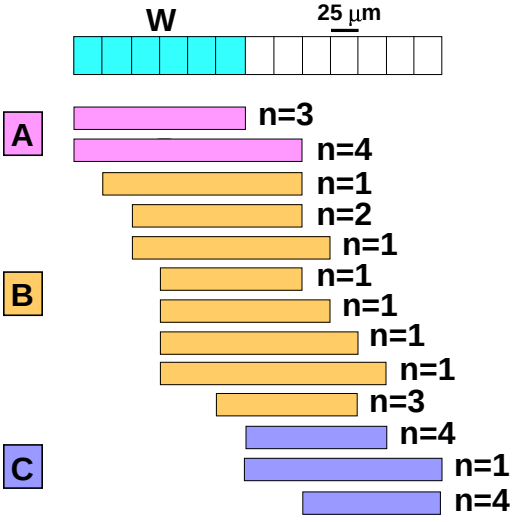


**Fig. 5.** Grafts of somatopleure in place of the neural tube. The scheme represents a transverse section of the wing somatopleure. Each division corresponds to 25 μm. The presumptive limb region, the W area, is in blue. The boxes underneath indicate the mediolateral limits of the strips of quail somatopleure transplanted in place of a segment of a neural tube. Strips of type A contained the entire zone W, strips of type B were taken from a region overlapping zone W and the lateral somatopleure, and strips of type C came from a region lateral to zone W.

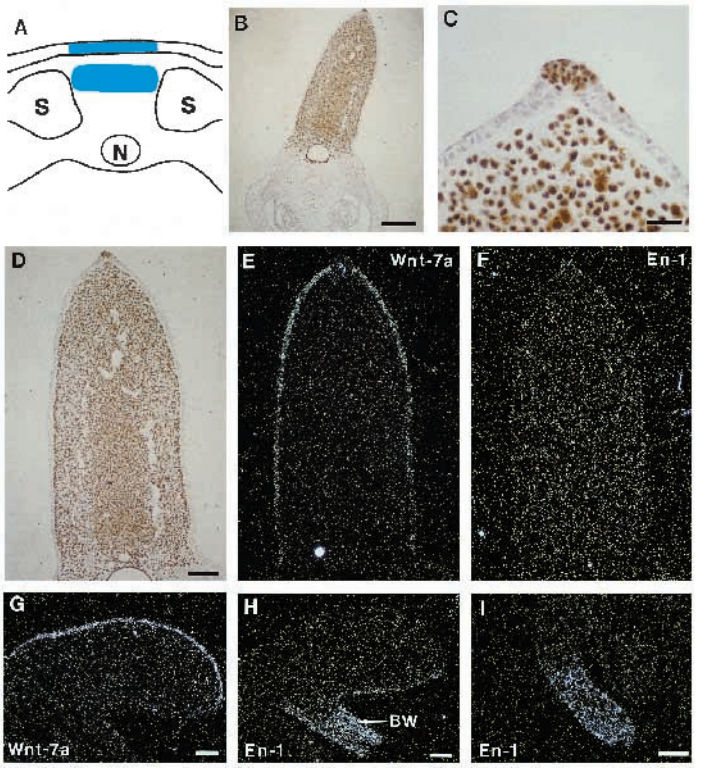


**Fig. 6.** Graft of quail wing somatopleure in place of the chick neural tube analyzed 48 hours after the operation. (A) Strips of quail somatopleure taken from the regions indicated in Fig. 5 are grafted in place of the neural tube in chick embryos. The notochord is left intact. (B-D) Different magnifications of a transverse section of a limb bud produced by the grafting technique described in A. The mesenchyme is mainly composed of quail cells (in brown). In the ectoderm, the quail cells are found around the dorsoventral midline of the AER (C). (E) A section adjacent to D was hybridized with *Wnt-7a*. In this limb bud, *Wnt-7a* is expressed in the entire ectoderm, with the exception of the AER. Compare with G, a transverse section of a control limb bud (stage HH22) in which*Wnt-7a* is only expressed in the dorsal ectoderm. (F) A section adjacent to D was hybridized with *En-1*, which is not detectable in the ectoderm and in the AER of the experimental limb bud. Compare with H, a transverse section of a control limb bud (stage HH 22) in which *En-1* is expressed in the ventral ectoderm and ventral half of the AER. (I) Dark-field photomicrograph of the same section as in F. *En-1* expression is detected in the ventral body wall (BW) ectoderm and mesoderm. Bars, 250 μm (B), 25 μm (C) and 100 μm (bar in D for D-F; G-I).

embryos in place of the neural tube either at the level of the wing (*n*=5) or the flank region (*n*=22; Fig. 6A). Embryos were analyzed 48 hours later. In all cases, even in those in which the grafted strip came from a region lateral to the presumptive limb region, a limb bud with its AER developed (Fig. 6B-D). With the exception of the AER, the ectoderm was always composed of chick cells which are thus derived from the paraxial ectoderm. The AER was composed of quail cells on its midline and of chick cells laterally, strikingly reproducing the pattern obtained in experiment 1 (Fig. 6C). In the wider grafts (200 μm), the relative contribution of the quail cells to the composition of the AER was greater, with in some instances the quail cells occupying almost the entire AER. An important difference, however, from the limb buds obtained in experiment 1 was that all the limb buds were bidorsal, as evidenced by the expression of *Wnt-7a* (Fig. 6E,G) or *Lmx-1* (not shown) on both sides and the total absence of *En-1* transcripts (Fig. 6F,H,I) whether the graft was implanted at the level of the wing or of the flank. These results confirm that the dorsoventral polarity of the presumptive limb region is not yet determined at late stage HH13 and suggest that the paraxial structures produce a dorsalizing factor.

Limb buds resulting from the same types of grafts (A, B and C) were analyzed 14-24 hours following the operation to study the distribution of the quail cells with respect to the DV interface. This was performed by comparing expression of the QCPN epitope and *Fgf-8* on adjacent sections, since at that stage, the AER is not yet visible or it is just beginning to appear (stage HH17-18; Todt and Fallon, 1984). Remarkably, in 14/18 cases, the quail cells and the *Fgf-8*-expressing cells were exactly the same, as shown in Fig. 7. In the four other embryos a few quail cells were distributed beyond the *Fgf-8* domain. Therefore, as was the case in isotopic grafts of zone W analyzed after an incubation of 24 hours, the DV interface of these early chimeric limb buds is completely derived from the transplanted somatopleural ectoderm. In chimeric limb buds analyzed a few more hours after the graft (stage HH19), the AER was entirely composed of quail cells (*n*=3; Fig. 7C). In normal limb buds of early stages, *En-1* is expressed in the ventral ectoderm, including the ventral half of the *Fgf-8*

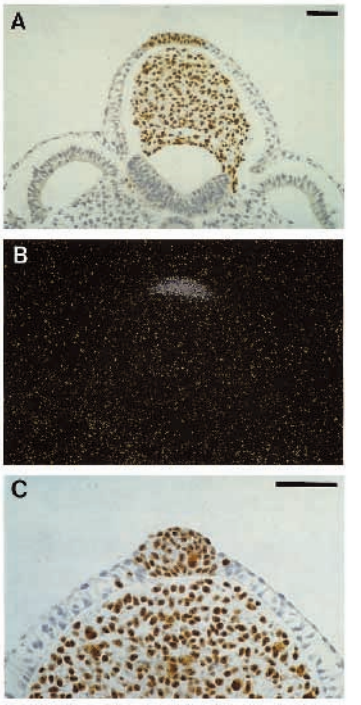


**Fig. 7.** Graft of quail somatopleure in place of a chick neural tube analyzed after 14-28 hours of incubation. (A) Transverse section of an experimental limb bud collected after 16 hours. The AER is not yet distinguishable. The quail cells are revealed by the anti-QCPN antibody. (B) Adjacent section of the one shown in A hybridized with *Fgf-8*. Note that the quail cells found in the ectoderm and the *Fgf-8*-expressing cells are the same. (C) Transverse section of an experimental limb bud collected 28 hours after the operation, once the AER has appeared. Note that the AER is exclusively composed of quail cells (in brown). Bars, 50 μm (A-C).

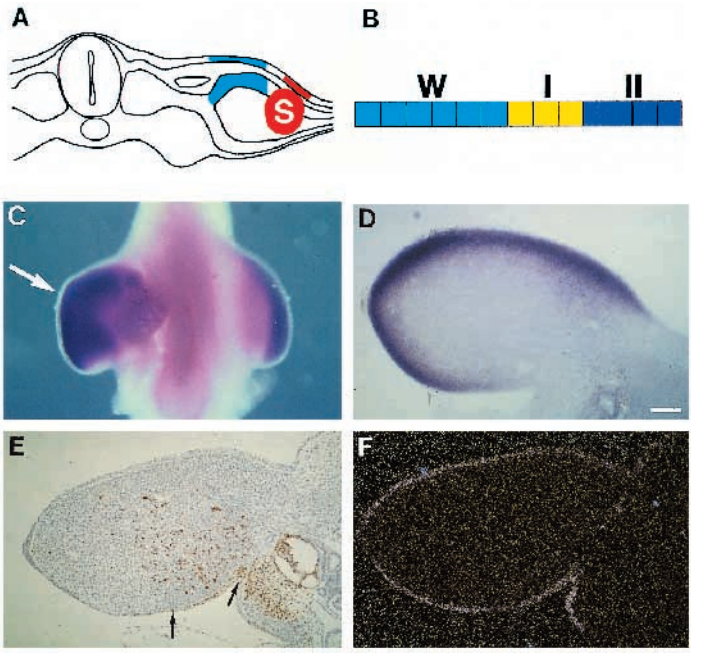


**Fig. 8.** Graft of somites laterally to the presumptive wing region. (A) Schematic transverse section of a late stage HH13 embryo illustrating the surgical operation. Zone W is in blue. (B) Scheme showing the regions in which the somites (S) were grafted. Each division corresponds to 25 μm. (C) Example of a bidorsal limb bud (indicated by an arrow) produced by grafting somites in zone II. *Lmx-1* expression is induced ventrally. (D) Transverse vibratome section of the experimental limb bud shown in C. (E) Distribution of the quail cells (in brown) in a bidorsal limb bud produced by grafting quail somites in a chick somatopleure. The quail cells (located between the two arrows) occupy the proximal third of the ventral ectoderm. (F) Adjacent section of the one shown in E hybridized with *Wnt-7a* which is induced in the ventral ectoderm. The *Wnt-7a*-expressing cells are of both chick and quail origins. Bar, 100 μm (D-F).

domain (not shown). *En-1* expression was not detected in the experimental limb buds, even in the dorsoventral interface (*n*=6; not shown).

The fact that the explants of type C yield a limb shows that the capacity to produce a limb at the level of somites 15-20 exceeds the real limb bud presumptive territory. The existence of an actual limb morphogenetic field in the lateral plate is thus demonstrated. Moreover, this indicates that the lateral somatopleural ectoderm has the competence to form an AER. In order to determine whether this is also the case for the paraxial ectoderm, we transplanted strips of wing somatopleural mesoderm overlaid by paraxial ectoderm taken between somites 15-20 in place of the neural tube. This resulted in the growth of limb buds composed of an AER (not shown). Therefore, at late stage HH13, the competence of the ectoderm to form an AER is not confined to a specific region along the DV axis, confirming results previously obtained by Kieny and Brugal (1971, 1977). The AER formation is thus entirely dependent on an induction from the mesoderm of zone W.

To further demonstrate that the paraxial structures produce a dorsalizing factor, rows of brachial somites covered with their own ectoderm were taken from late stage HH13 quail embryos and grafted at different positions laterally to the presumptive wing region of stage matched chick embryos (Fig. 8A,B). Grafts of somites in a region of about 75 μm (zone I; Fig. 8B) located immediately laterally to the W region (*n*=12) induced a variety of abnormalities in the limb buds (atrophy, duplication along the dorsoventral axis, and duplication along the anteroposterior axis). One of these abnormal limb buds was bidorsal, as shown by the expression of *Wnt-7a*. In contrast, somites grafted more laterally (zone II, Fig. 8B) did not perturb the morphology of the limb buds, which in 6/7 cases had a bidorsal polarity (Fig. 8C-F). In order to determine whether there was a correlation between the presence of quail cells in the ventral ectoderm and the induction of *Wnt-7a*, the expression of the QCPN epitope and of *Wnt-7a* were compared on adjacent sections (*n*=4). Quail cells were found proximally in the ventral ectoderm and they always expressed *Wnt-7a*. However, *Wnt-7a* expression was not restricted to these cells, as it was also induced in the chick cells of the ventral ectoderm

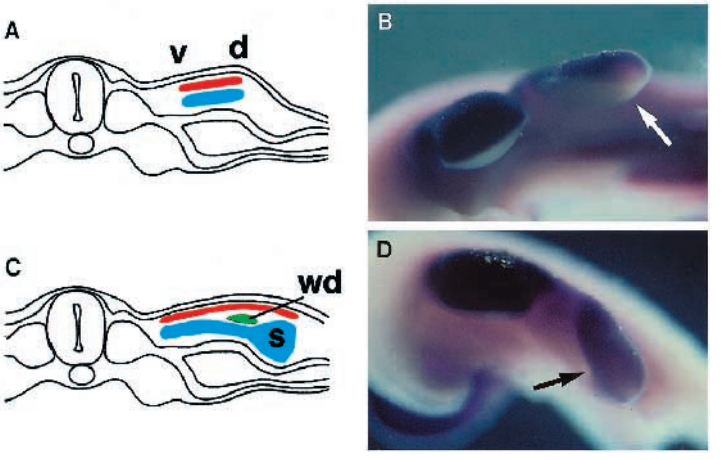


**Fig. 9.** Graft of somatopleure to the flank. (A) Graft of the mesoderm (in blue) and of the ectoderm (in red) of zone W under the flank ectoderm in a reversed dorsoventral orientation. (B) The limb bud produced by the graft described in A is indicated by the arrow. *Lmx-1* expression pattern is the same as in ipsilateral control limb buds. (C) Graft of zone W and of the associated paraxial structures under the flank ectoderm in a reversed dorsoventral orientation. wd, Wolffian duct; s, somites. (D) The limb bud produced by the graft described in C is indicated by an arrow. *Lmx-1* is expressed on both side of this limb bud. Only the dorsal side of the control limb bud can be seen in this picture.

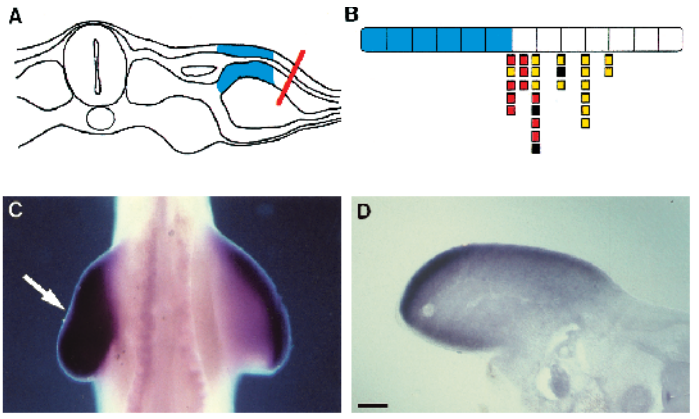


**Fig. 10.** Insertion of nuclepore filters laterally to zone W can induce bidorsal limb buds. (A) Schematic transverse section of a late stage HH13 embryo illustrating the surgical operation. Zone W is in blue. (B) Positions of insertion of filters along the mediolateral axis of the somatopleure. Each division corresponds to 25 μm. Zone W is indicated in blue. Each square corresponds to a single case. The red squares indicate that the insertion induced *Lmx-1* ventrally; the yellow squares indicate that the filter had no effect on dorsoventral patterning; and the black squares indicate that the induction was weak. (C) Example of a bidorsal limb bud (indicated by an arrow) resulting from the insertion of a filter immediately laterally to the W region. (D) Transverse vibratome section of the limb bud shown in C. Bar, 100 μm (D).

(Fig. 8E,F). As shown for grafts of somatopleure in place of the neural tube, the expression of *Wnt-7a* and of *En-1* was mutually exclusive. In a second set of experiments, somites were grafted without their ectoderm in zone II. In 2/5 cases, *Lmx-1* was induced in the ventral mesenchyme of the limb bud. We conclude that the somites associated with their overlying ectoderm are a powerful inducer of dorsal limb polarity. The somitic mesoderm, however, by itself can exert a similar influence.

### Grafts of somatopleure to the flank

Saunders and Reuss (1974) found that limbs produced by grafting stage HH12/13 wing somatopleural mesoderm under the flank ectoderm have the dorsoventral polarity of the graft. In contrast, Kieny (1971) found that the dorsoventral polarity of these grafts was determined by their environment. We repeated this experiment. The W region ectoderm and mesoderm were grafted ipsilaterally under the flank ectoderm in a reversed dorsoventral orientation and the embryos were analyzed 48 hours later. In all cases ($n$=7), the limb buds adopted the dorsoventral polarity of the ipsilateral host limb buds, as shown by *Lmx-1* expression (Fig. 9A,B). This result shows that the flank environment can also polarize stage 13 wing somatopleure. In a second series of grafts, both the somatopleure and the mesonephros were inserted under the flank ectoderm, again in a reversed dorsoventral orientation ($n$=7). The resulting limb buds also had the same dorsoventral polarity as the ipsilateral host limbs (not shown). The growth of the limb buds examined in these two series of experiments was always directed ventrally. In a last set of experiments, the somatopleure, the mesonephros and the somites were grafted to the flank in a reversed dorsoventral orientation (Fig. 9C). All the limb buds resulting form these grafts were bidorsal ($n$=7; Fig. 9D). The direction of their growth was neutral in 5/7 cases and dorsal in the remaining cases. These results confirm that the somites dorsalize the limb bud and suggest that the mesonephros is not involved in that process.

### Separation of the W region from the lateral somatopleure gives rise to bidorsal limb buds

In order to explore the possibility that the lateral somatopleure produces a ventralizing factor, we inserted a nuclepore filter (0.4 μm pore diameter) at different positions laterally to the W region (Fig. 10A,B). Insertion of filters in a region of 50 μm located immediately lateral to the W region gave rise to bidorsal limb buds in 13/19 cases (68%), as shown by *Lmx-1* expression (Fig. 10C,D). Filters inserted further laterally did not have any effect on dorsoventral patterning ($n$=8). Insertion in the W region resulted in severely abnormal limb buds. These results are consistent with the existence of a ventralizing factor produced by the lateral somatopleure. The fact that permeable obstacles had an effect on the limb bud DV polarity suggests that this ventralizing signal is non-diffusible. Alternatively, the filters could interfere with a signal which acts over a short distance.

## DISCUSSION

When it first appears at stage HH16, the wing bud is already polarized along the DV axis. To study the mechanisms leading to the establishment of this polarity, we decided to focus our attention on an earlier stage (HH13). Using the quail-chick chimera system, we first showed that the presumptive wing mesoderm occupies the medial half of the somatopleure, a zone that we designate as the W region. The corresponding ectodermal area, however, will only give rise to the AER. The dorsal limb bud ectoderm originates from the ectoderm overlying the somites and the intermediate mesoderm. The ventral limb bud ectoderm comes from the ectoderm overlying the somatopleural mesoderm (Fig. 3). Using this information, we changed the environment of the presumptive region by several experimental paradigms. We provide evidence that the DV information does not reside in the presumptive limb territory itself at that stage but is determined by its environment. We show that the polarization of the wing somatopleure results from the production of a dorsalizing factor by the somites and of a ventralizing factor by the lateral somatopleure which operate between stage HH13 and HH15.

### Delineation of the DV interface and involvement of the somites in patterning the dorsoventral axis of the limb bud

The induction of limb development depends on a signal produced by the somites and by the mesonephros around stage HH13/14 (Pinot, 1970; Kieny, 1971; Stephens and McNulty, 1981; Geduspan and Solursh, 1992; Stephens et al., 1993). IGF-1 and FGF-8, which are both expressed by these structures, appear to play an important role in this inductive process (Crossley et al., 1996; Dealy and Kosher, 1996). Geduspan and

Solursh (1992) have shown that, at stage HH14-16, the presumptive limb mesoderm occupies the medial half of the somatopleure and that its lateral half can also give rise to a limb when grafted immediately lateral to the mesonephros. Our fate map and our grafts of somatopleure in place of the neural tube support these results, showing that at these early stages the entire somatopleure located between somites 15 and 20 constitutes a morphogenetic field larger than the actual presumptive wing territory. Any region of this field can become a wing if subjected to the appropriate inducing influences. A similar conclusion has been reached for an amphibian (Amano, 1960).

The present work provides an important clue about how the DV interface is specified. The chimeric limb buds that we produced at late stage HH13 either by isotopic grafts of wing somatopleure or by grafting somatopleure strips in place of the neural tube, showed that, initially the DV interface, including the early stage AER, is entirely derived from the ectoderm overlying the presumptive limb mesoderm. With further development of the limb bud, some additional cells of the dorsal and ventral ectoderms are incorporated in the flanks of the AER. Classical studies have shown that the somatopleural mesoderm induces the AER (Kieny, 1960; Saunders and Reuss, 1974; Carrington and Fallon, 1984). Our data suggest that, at stage HH13/14, the mesoderm of zone W induced by the somites and the mesonephros specifies the entire overlying ectoderm to acquire its DV interface identity (Fig. 11A). One consequence of the induction by the mesoderm appears to be the onset of cell death (Vaahtokari et al., 1996) and/or a decrease in cell proliferation, as the prospective AER expands much less than do the ectoderms overlying the intermediate mesoderm and the lateral somatopleure, which will cover the dorsal and the ventral aspects of the limb, respectively.

A series of classical experiments carried out in the amphibian *Amblystoma* have shown that while the anteroposterior polarity of the presumptive limb region is determined as early as at the completion of gastrulation (Detwiler, 1933), its dorsoventral polarity is acquired much later, shortly before the appearance of the limb bud (Harrison, 1918, 1921; Ruud, 1926; Swett, 1927; Hollinshead, 1936). In the chick, there is also some evidence that the anteroposterior axis is determined early, possibly as early as stage HH10 (Hornbruch and Wolpert, 1991). However, the stage at which the presumptive limb acquires its DV polarity is controversial (Kieny, 1971; Saunders and Reuss, 1974).

In order to address the question of the determination of the DV axis of the limb bud, we used several experimental procedures to modify the environment of stage HH13 presumptive limb mesoderm. When the presumptive wing region was rotated by 180° or when it was transplanted to the flank with a reversed dorsoventral orientation, a limb bud developed which had the dorsoventral polarity of a non-rotated or non-inverted control limb as shown by *Lmx-1* expression. In other words, at stage HH13, the dorsoventral polarity does not reside in the presumptive limb mesoderm but is determined by its environment. To see whether the paraxial structures play a role in the induction of the DV polarity, we flanked the presumptive limb region by two rows of somites, either by transplanting it in place of the neural tube, by grafting a row of somites laterally in the lateral plate mesoderm or by inserting the somatopleure with the paraxial structures in a reversed dorsoventral orientation under the flank ectoderm. These manipulations resulted in the growth of bidorsal limb buds, suggesting that the somites produce a dorsalizing factor. Similar experiments performed in amphibians also showed that the paraxial structures play a role in the polarization of the dorsoventral axis (Swett, 1938). Therefore, the presumptive limb bud is patterned by at least two paraxial signals: the first signal, produced by the mesonephros and the somites, is responsible for limb induction and the second signal, provided by the somites, dorsalizes the limb bud. One possible explanation for Saunders and Reuss' (1974) results is that portions of somites or of presomitic mesoderm were included in their graft.

### Role of the ectoderm in the dorsalization of the presumptive limb mesoderm

Experiments carried out by a number of investigators have clearly indicated that from stage HH15/16 on, the ectoderm of the limb bud can impose its DV polarity upon that of the autologous mesoderm. The mechanisms by which the somites can polarize the presumptive limb ectoderm remain unclear. One possiblity is that, between stage HH13 and HH15, the somites directly polarize the mesoderm of zone W, which could then transfer the dorsoventral information to the overlying ectoderm (Fig. 11B).

Another possibility is that, between stage HH13 and HH15, the somites specify the presumptive dorsal ectoderm, which overlies the paraxial and the intermediate mesoderms, to acquire a dorsal identity (Fig. 11C1). The morphogenetic movements of this ectoderm layer would then carry the dorsal information over the wing mesoderm (Fig. 11C2). When we grafted quail somites and their overlying ectoderm laterally to the presumptive wing region of chick hosts, we observed no correlation between the presence of quail cells in the ventral ectoderm of the limb bud and the induction of *Wnt-7a*. Therefore, the paraxial mesoderm has induced the adjacent chick ectodermal cells to acquire a dorsal identity. The same explanation could account for the dorsalizing effect of somites deprived of their ectoderm. These observations are consistent with a model which predicts that there are two dorsalizing factors. First, a factor is produced by the somites, which induces the presumptive dorsal ectoderm to acquire a dorsal identity. This ectoderm then proliferates over the limb mesoderm and secretes the second factor, probably WNT-7a, which dorsalizes the wing bud.

Recombination experiments at stage HH14, in which dorsoventrally reversed ectoderm resulted in wings with mesodermal dorsoventral polarity, led Geduspan and MacCabe (1987, 1989) to propose that the presumptive limb mesoderm transfers the dorsoventral information to the overlying ectoderm at the limb budding stage. As these authors did not specify the boundaries of their grafts and in what orientation the recombinant tissues were grafted to the flank, it is difficult to interpret their results. Nevertheless, they raise the possibility that the presumptive ectodermal territories of the limb bud are not yet determined at stage HH14. The fact that a graft of somites laterally to the W region can program the presumptive ventral ectoderm to acquire a dorsal identity would also support this view.

In the limb regions, the ectoderm can be divided into two domains along the dorsoventral axis. (1) A dorsal one overlying the axial organs, the paraxial mesoderm and the

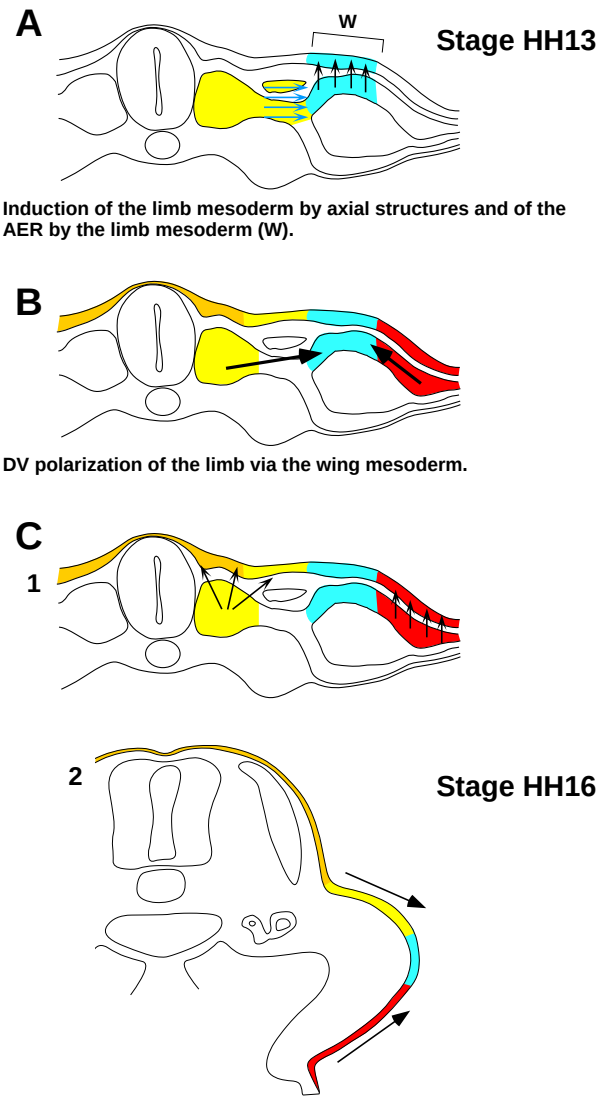


**Fig. 11.** Signals patterning the presumptive limb bud. Schematic transverse sections of late stage HH13 (A-C1) and of stage HH16 (C2) embryos. (A) At stage HH13/14, the mesonephros and the somites induce the mesoderm of zone W (in blue) which then specifies the overlying ectoderm to acquire a DV interface identity. (B,C) Alternative pathways to specify DV axis in the limb bud. (B) The dorsalizing and ventralizing signals act on the mesoderm of zone W. (C1). The somites and the lateral somatopleure act on the future dorsal and ventral limb ectoderm. (C2) The induced ectoderms grow over the budding limb and carry their dorsal and ventral identity that they secondarily impose to the limb mesoderm.

dorsal aspect of the limb bud. This dorsal ectoderm expresses *Wnt-7a* (Dealy et al., 1993; our unpublished results). (2) A ventral one which is composed of the ectoderm of the presumptive body wall and of the ventral aspect of the limb bud. This ventral domain expresses *En-1* (Davis et al., 1991; Gardner and Barald, 1992). The two domains meet at the AER. Our observation that the somites can induce *Wnt-7a* in the ventral ectoderm suggests that they are also involved in generating *Wnt-7a* expression in the axial and paraxial ectoderm.

### Specification of the limb bud ventral identity

The insertion of filters laterally to the W region gave rise to bidorsal limb buds. These filters are likely to interfere with the propagation of a signal produced by the lateral somatopleure. In the absence of this ventralizing factor, the presumptive ventral limb bud would acquire a dorsal identity. Therefore, polarization along the DV axis would result from the antagonistic actions of two signals: a dorsalizing one produced by the somites and a ventralizing one by the lateral somatopleure. These two signals could act through similar mechanisms, namely by directly polarizing the mesoderm of zone W or by inducing their respective overlying ectoderms (Fig. 11B,C). In the latter case, the filters would produce bidorsal limb buds by interfering with the morphogenetic movements of the lateral somatopleural ectoderm.

### Relationship between the establishment of the limb bud DV polarity and the formation of the AER

In the chick mutant *limbless*, limb buds develop normally until stage HH19. After that stage, no AER forms and the whole bud degenerates (Prahlad et al., 1979; Carrington and Fallon, 1988). *Fgf-8* and *En-1* transcripts are not detectable in these limb buds and *Wnt-7a* is expressed in the entire limb bud ectoderm (Ros et al., 1996; Noramly et al., 1996; Grieshammer et al., 1996). Meinhardt (1983) proposed that the juxtaposition of a dorsal and of a ventral domain is a prerequisite for the formation of the AER. The fact that in *limbless* the limb buds are characterized by the absence of both a dorsoventral juxtaposition and an AER supports this hypothesis. Our finding that an AER can form in a bidorsal context (see also Carrington and Fallon, 1986) would instead suggest that establishment of DV polarity and AER formation occur independently. It is possible, however, that the main function of the markers used in this study, *En-1*, *Wnt-7a* and *Lmx-1*, is to confer a dorsal or a ventral identity to the tissues in which they are expressed and that AER specification results from the dorsoventral juxtaposition of other genes in the ectoderm and/or mesoderm of zone W. Our results also do not necessary imply that DV interface specification is solely based on the interaction between the ectoderm and the mesoderm of zone W. For instance, signaling between the presumptive DV interface and the adjacent ectoderms could contribute to the delineation of the limb ectoderm territories.

We could not detect *En-1* transcripts in the AER of bidorsal limb buds. This might indicate that *En-1* expression in the ventral AER is dependent upon the presence of an adjacent ectoderm which has a ventral identity. In limb buds of *En-1* mutant mice, the ventral border of the AER is shifted ventrally and proximally, whereas the dorsal border is located normally (Loomis et al., 1996). It is noteworthy that the morphology of the AER of bidorsal limb buds produced in our experiments was normal even though *En-1* expression was not detected. The basis for this discrepancy remains unclear.

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